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FINAL REPORT

Study Title

In Vivo Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Test Article

N,N,N',N'-tetramethyl ethanediamine (TMEDA)

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Laboratory Project I.D.

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14. ABSTRACT

N,N,N?,N?-tetramethyl ethanediamine (TMEDA, 99.86% pure) was tested for its potentials to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and female CD-1 mice according to OECD TG 474 in compliance with Good Laboratory Practice. Male and female mice were dosed orally at 62.5,125 and 250 mg/kg. Animals were euthanized approximately 24 and 48 hrs after dosing. The percent of polychromatic erythrocytes (PCE) and frequency of micronucleated polychromatic erythrocyte (MPCE) were determined at 24 and 48 hrs. Cytotoxicity was assessed by scoring the number of PCE and norchromatic erythrocyte (NCE) in first 200 erythrocytes for each animal. There were no statistically significant increases in the number of MPCE in the treated groups when compared to the concurrent vehicle control group. These results indicate that TMEDA was negative in the in vivo mouse micronucleus assay and the compound was not clastogenic.

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STUDY DIRECTOR'S COMPLIANCE STATEMENT

Study No. 0977-1521
Sponsor's Test Article I.D.: N,N,N',N'-tetramethyl ethanediamine (TMEDA)

The study described in this report was conducted in compliance with the following test guidelines:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Section 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002, (1).

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997, (2).

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996, (3).

The study described in this report was conducted in compliance with the following Good Laboratory Practice standard, except as indicated in the last paragraph:

United States Food and Drug Administration Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2006, (4).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005, (5).

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 Nohsan, Notification No. 6283, October 1, 1999, (6).

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997, (7).

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984 (8).

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998, (9).

The strength and stability of the test article, dosing solutions and controls, under the experimental conditions, were not determined.

Signature: _____ Date: 6-19-08

Jian Song, Ph.D.
Study Director

QUALITY ASSURANCE UNIT'S STATEMENT

Study No. 0977-1521

Sponsor's Test Article I.D.: N,N,N',N'-tetramethyl ethanediamine (TMEDA)

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 01/15/08

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
03/11/08	Weighing the Test Article Positive Control	03/11/08	03/12/08
04/18/08	Work Book Audit	04/19/08	04/22/08
04/22/08	Draft Report Audit	04/22/08	04/22/08
06/13/08	Final Report Audit	06/13/08	06/16/08

Signature: Vian Lambert Date: 06/19/08

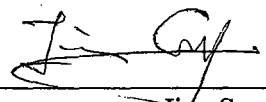
Vian Lambert, B.S.
Quality Assurance Manager

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Shambhu K Roy* and Jian Song, Ph.D. Study Directors for In Vivo Micronucleus Studies, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Draft Report on this study was written by Dr. Jian Song and Karen S.K. Shore. Dr. Song released the report on June 19, 2008.

Signature



Jian Song, Ph.D.
Study Director

6-19-08
Date

* Dr. Roy was the Study Director for this assay until his departure from SITEK's employ on February 29, 2008, whereupon Dr. Jian Song assumed the position of Study Director. Dr. Roy departed after the protocol was signed, but prior to the experimental initiation. Dr. Song supervised the actual conduct of this study.

ABSTRACT

The test article, N,N,N',N'-tetramethyl ethanediamine (TMEDA, 99.86% pure), was tested for its potential to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and female CD-1 mice. Water was used as the vehicle control. The test article and vehicle control were administered by oral gavage. Three male and three female mice per dose group were used in the Range Finding Test. Five male and five female mice per dose group per harvest time were used in the Micronucleus Assay

The MSDS of TMEDA indicated that oral rat LD50 is 268 mg/kg. The dose levels selected for the Range Finding Test in mice were 300, 200, 100, 50, 10 and 5.0 mg/kg body weight. A marked decrease in activity was observed at 200 and 300 mg/kg in both males and females on Day 1. On Day 2 one male died in the 300 mg/kg group and the remaining males and all females at 300 mg/kg were less active than the vehicle control animals. On Day 3 and 4, no abnormal clinical signs were observed in any group.

Test article doses of 62.5, 125 and 250 mg/kg for the Micronucleus Assay were selected based on the results of the Range Finding Test. The highest dose was selected to exclude lethality in accordance with U.S. E.P.A., OECD and ICH guidance documents. The positive control group received a single, oral gavage dose of cyclophosphamide (CP) at 80 mg/kg body weight. Animals were euthanized approximately 24 and 48 hours after dose administration. Positive controls were included in the 24-hour harvest only.

In the Micronucleus Assay, the percentage of polychromatic erythrocytes (PCE) and frequency of micronucleated polychromatic erythrocytes (MPCE) were determined at approximately 24 and 48 hours after the dose administration. Two thousand PCE per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCE and normochromatic erythrocytes (NCE) in the first 200 erythrocytes for each animal. There were no statistically significant increase in the number of MPCE in the treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups or SITEK's historical vehicle control data. Reduction in the percentage of PCE was used as an indication of toxicity. In the 62.5 mg/kg group, 48 hours after treatment the reduction of PCE was 21.2% in male mice compared with the vehicle control. In all other groups, no reduction (more than 20% versus that of the vehicle control) in the percentage of PCE was observed.

The frequency of MPCE in each treatment group was compared to that in the respective vehicle control using a one-tailed Student's T-test. The vehicle and positive control groups met the criteria for a valid test. The data from all of the dose groups treated with TMEDA, at both bone marrow harvest time points, were not significant when compared to the vehicle control group. Therefore, the results of this assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test

results, the test article was negative in the Micronucleus Assay. It was concluded that the test article did not cause chromosome damage *in vivo* and, therefore is not considered to be a clastogenic agent.

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INTRODUCTION

This study was conducted by Shambhu K. Roy, Ph.D., Jian Song, Ph.D., Hussain Shaffi, B.S., Weiyu Xie, M.D., Karen S. K. Shore, B.A., Adrienne Parker, B.S. and Ann Nguyen, A.A., at SITEK Research Laboratories from March 4, 2008 to March 30, 2008. The experimental procedures used to perform this study were essentially those of J.A. Heddle (10), W. Schmid (11), K.H. Mavournin, et al (12), M. Hayashi, et al (13) and R.R. Tice and M.D. Shelby (14).

The purpose of this study was to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes (MPCE) in mouse bone marrow cells (10, 11). Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (12-14).

MATERIALS

TEST ARTICLE

1. Name:	N,N,N',N'-tetramethylethanediamine (TMEDA)
2. CAS No. :	110-18-9
3. Provided By:	USA RDECOM, AMSRD-MSF
4. Batch/Lot No.:	10588KD
5. Physical Description:	Clear liquid
6. Shipping Conditions:	Room Temperature
7. Date Received at SITEK:	January 10, 2008
8. Storage Conditions:	Refrigerated (1-5°C)
9. Purity:	99.86%
10.Expiration Date:	N/A

A Certificate of Analysis for the test article is presented in Appendix IV .

CONTROL SUBSTANCES

Positive Control

Cyclophosphamide (CP), which induces micronucleus formation, was used at 80 mg/kg body weight (8.0 mg/mL x 10 mL/kg) by oral gavage in this study. The information on the CP used in this study is provided below:

1. Source:	Sigma Chemical Company
2. CAS No.:	6055-19-2
3. Lot No.:	075K1661
4. Purity:	99.7% by HPLC
5. Storage Conditions:	Refrigerated (1-5°C)
6. Expiration Date:	May 2, 2011

Sterile water (Baxter Lot No. C715987) was used to dissolve the positive control. The expiration for this lot was July 2008.

Vehicle Control

Water was used as the vehicle control.
The information on the water used in this study is provided below:

1. Source:	Baxter
2. CAS No.	7732-18-2
3. Lot No.:	C715987
4. Storage Conditions:	Refrigerated (1-5°C)
5. Expiration Date:	July 2008

TEST ANIMALS

Approximately 42-day-old, male and female, CD-1 mice were obtained from Harlan Sprague Dawley, Inc. (Frederick, MD)

The animals were housed three or five animals per cage during the study. Hardwood chip bedding, free of injurious substances, was used. The animals received Purina Certified Rodent Diet (Code No. 5002C, Lot No. NOV 18 07 3C for Range Finding Test, Lot No. DEC 30 07 1A for Micronucleus Assay, Brentwood, MO) and fresh tap water *ad libitum*. Levels of contaminants present in the food and water were within acceptable levels. Bedding was changed at least twice per week.

The animals were quarantined for at least 7 days for the Range Finding Test prior to dose administration. The animals were observed each day and the observations and the temperature and humidity of the animal room were recorded in the study notebook. Throughout the trials, the animal room was maintained at 17-23°C and 17-54% humidity. A 12-hour diurnal light cycle was employed.

A total of 42 mice (21 males, 21 females) were used for the Range Finding Test. A total of 90 mice (45 males, 45 females) were used for the Micronucleus Assay.

EXPERIMENTAL PROCEDURE

DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study were entered into the study notebook (0977-1521). The study notebook also includes the original protocol, study report copies, and all relevant communications with the Sponsor.

TEST SYSTEM IDENTIFICATION

All of the animals to be dosed received an ear tag with a number unique to the particular study. All of the cages were assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director, and the animal study proposal number (06-02-13) approved by SITEK's IACUC. The microslides were labeled with SITEK's study number, the last three digits of the animal number as a code number, and the date of slide preparation.

SOLUBILITY TEST

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test was performed.

The test article was tested for its solubility/miscibility in deionized, distilled water. A nonviscous liquid will be tested for miscibility in weight per volume. The solubility/ miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test would consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article was dissolved or until 1.5 mL of vehicle had been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in aliquots of 0.5 mL until 5.0 mL had been added. The volume of vehicle required for complete dissolution, and any additional observations, would be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle would be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test would be conducted. 1.5 mL of vehicle in 0.1 mL increments would be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in 0.5 mL increments until 5.0 mL had been added. The resulting solution would be thoroughly mixed and observed for miscibility. The test article would be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations would be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article would be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data were available, the solubility/miscibility test would not be performed.

RANDOMIZATION OF TEST ANIMALS

Upon arrival, the animals were randomly assigned to clean, polycarbonate cages. All of the animals were ear tagged with a number unique to the study. In the Range Finding Test, the animals were assigned to experimental groups (three animals per sex), without regard to body weight, prior to dose administration.

In the Micronucleus Assay, the animals were weighed and placed into weight groups. Each weight group spanned 1 gram. Using a computer-generated random matrix, the animals then were assigned sequentially from the weight groups to randomized cages corresponding to the treatment groups (five animals per sex per group).

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

The stock solutions for the Range Finding Test and the Micronucleus Assay were prepared as specified in the dilution scheme which was kept in the study notebook. The specified amount of test article was weighed and the required volume of water was added to reach the highest stock concentration. The remaining concentrations were made by subsequent dilution.

The dosing solutions were prepared by SITEK study personnel just prior to treatment. In all treatments, the amount of vehicle administered to the animals was limited to a level which would have no significant toxic effect. The strength and stability of the test article and the test article dosing solutions under the experimental conditions was not determined by SITEK Research Laboratories.

TREATMENT PROCEDURE

The animals were treated with a single dose by oral gavage. The positive control (CP) was also given as a single dose by oral gavage. The total dose volume administered to the animals was 10 mL/kg body weight for each test article dose level and vehicle control. 10 mL/kg body weight was used for the positive control.

RANGE FINDING TEST

In the Range Finding Test, treatment groups of three mice per sex were treated at six test article dose levels of 5.0, 10, 50, 100, 200 and 300 mg/kg body weight and one group was treated with the vehicle control.

The animals were observed for 3 days following dose administration for treatment-related clinical signs and/or death. Body weights were checked on the day of dosing (Day 1) and at the end of the 3-day observation period (Day 4). At the end of the 3-day observation period, the animals were euthanized. The body weight data were entered into the computer to calculate the mean body weight and percent change in the mean body weight, using a MS Office Excel 2003 spreadsheet program. Clinical signs were entered into the computer in tabular form to demonstrate frequency. Doses for the Micronucleus Assay were selected based on the results of the Range Finding Test.

MICRONUCLEUS ASSAY

The Micronucleus Assay was performed at 62.5, 125 and 250 mg/kg body weight based on the results of the Range Finding Test.

The animals were randomized and placed into treatment groups of five mice per sex. Two such treatment groups were designated for each of the test article dose levels and the vehicle control per harvest (a total of 80 mice). One group was designated for the positive control (10 mice). Water and CP were used as the vehicle control and positive control, respectively. The dose volume was 10 mL/kg body weight for the test article, the vehicle control and the positive control.

The mice were euthanized by CO_2 asphyxiation approximately 24 or 48 hours after the dose administration. Five male and five female mice were euthanized from each test article dose level and the vehicle control at each harvest time. Five male and five female mice treated with the positive control were euthanized 24 hours after treatment.

After each animal was euthanized, the groin area was cleansed with 70% ethanol, and the femurs were exposed by cutting into the skin and muscle of the thighs. The femurs were separated just above the kneecaps, and the heads of both femurs were removed with scissors. The bone marrow from the femurs was flushed into a disposable culture tube, containing 1.0 mL of fetal bovine serum, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes were centrifuged at 800 rpm for 5 minutes. The supernatant was removed, leaving approximately 0.1 mL of serum above the cell pellet. The cells were resuspended by flicking the tube until a homogeneous suspension was observed.

A small drop of the cell suspension was placed on the unfrosted end of a clean microscope slide and spread along the length of the slide. The slides were allowed to air dry, then fixed in methanol for 15 minutes and allowed to air dry again. The slides were stained in Wright-Giemsa stain for 2-4 minutes, then rinsed in distilled water, allowed to air dry completely, and mounted in Cytoseal using #1 cover glasses. The backs of the slides were cleaned with methanol.

The slides were scored "blind" in order to avoid bias on the part of the scorers. First, the number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) among 200 erythrocytes (PCE + NCE) per animal was determined. The number of micronucleated polychromatic erythrocytes (MPCE) then was determined for 2000 PCE per animal (2).

STATISTICAL ANALYSIS

The data from the score sheets were consolidated into summary sheets and entered into a computer, using an MS Office Excel 2003 spreadsheet program. The dose group means were calculated for the percentage of PCE as well as the frequency of MPCE. A significant reduction in percent PCE (more than 20% versus that of the vehicle control) was used as an indication of toxicity.

Data were analyzed separately for male and female animals. The frequency of MPCE in each dose group was compared to that in the respective vehicle control group using a one-tailed Student's t-test (15). The data from female mice was compared to historical vehicle control data due to the number of MPCE in the concurrent vehicle control was 0 which is unusually low. An Excel 2003 statistical package was used to calculate p values for the t-test. The results were considered significant if the p value was ≤ 0.025. Statistical analysis was not performed on values that were lower than or equal to those of the respective vehicle control. The Cochran-Armitage Test (trend test) (16) was used to analyze

trends if the Student's t-test showed a positive result. The trend test was considered significant if the p value was ≤0.05. For this study no trend test was needed.

CRITERIA FOR A VALID ASSAY

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed 10.

2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.

3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

EVALUATION OF TEST RESULTS

Positive Response

The test article was considered to have caused a positive response in this assay if:

1. The test article showed a positive dose-response trend and a statistically significant increase ($p \leq 0.25$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article caused a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may have been compared to historical vehicle control data. For this study the data from both 24 and 48-hour harvest in female mice have been compared to historical vehicle control data (see Table 11 and Table 13).

2. In the event there was no positive dose-response trend, at least two consecutive test doses must have produced a statistically significant increase in the number of MPCE.

Negative Response

The test article was considered to have caused a negative response if none of the test doses showed a statistically significant increase in the number of MPCE when compared to the vehicle control.

Equivocal Response

The test article was considered to have caused an equivocal response if the test article induced a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend. In such a case, a repeat assay would have been performed only with the approval of the Sponsor.

ARCHIVES

The raw data, protocol, protocol amendments, protocol deviations, documentation, and a copy of the Final Report, and an electronic file containing the data tables and Final Report of the study, will be maintained for ten years in SITEK Research Laboratories' archives located at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY TEST

The test article, N,N,N',N'-tetramethyl ethanediamine (TMEDA, 99.86% pure), was a clear liquid and a miscibility test was performed. The information specifying 99.86% purity of TMEDA was obtained from Sigma-Aldrich, Inc. The test article solution was miscible with the vehicle (water) at 414.6 mg/mL forming a clear solution.

RANGE FINDING TEST

In the Range Finding Test, the animal body weight range on the day of dose administration was 30-37 grams for the males and 24-29 grams for the females. After a single administration of TMEDA, the changes in the mean body weight for each dose group, which occurred by the end of the 3-day observation period following dosing, are presented in Table 1 (Appendix I). The data indicate that by the end of the 3-day observation period, No reduction of greater than 3.6% in the mean body weight was observed in any dose group of either sex but one male mouse at 300 mg/kg body weight group was found dead on Day 2.

Some abnormal clinical signs were observed in the 3-day observation period. The data are presented in Tables 2-9 (Appendix I).

MICRONUCLEUS ASSAY

Based on the toxicity results from the Range Finding Test, the doses for the Micronucleus Assay were 62.5, 125 and 250 mg/kg body weight. In the Micronucleus Assay, the animal body weight range on the day of dose administration was 31-35 grams for the males and 24-31 grams for the females.

The percentage of PCE and the MPCE frequency were determined from bone marrow preparations from animals euthanized approximately 24 and 48 hours after the dose administration. Individual results for the dose levels and vehicle groups in the Micronucleus Assay for the animals sacrificed approximately 24 and 48 hours after treatment are presented for the males in Tables 10 and 12, respectively, and summarized in Table 14 and for the females in Tables 11 and 13, respectively, and summarized in Table 15 (Appendix I).

Reduction in the percentage of PCE was used as an indication of toxicity. In the 62.5 mg/kg group, 48 hours after treatment the reduction of PCE was 21.2% in male mice compared with the vehicle control. In all other groups, no reduction (more than 20% versus that of the vehicle control) in the percentage of PCE was observed. The ranges of the mean numbers of MPCE in 2000 PCE from this assay are summarized below:

	Mean Numbers of MPCE in 2000 PCE in Males	
Treatment	24 Hours	48 Hours
Vehicle Control	0.6	0.8
Test Article Doses (62.5-250mg/kg body weight)	0.4-0.8	0.0-2.2
Positive Control-CP (80 mg/kg body weight)	28.8*	N/A

	Mean Numbers of MPCE in 2000 PCE in Females	
Treatment	24 Hours	48 Hours
Vehicle Control	0.0**	0.0
Test Article Doses (62.5-250 mg/kg body weight)	0.0-0.6	0.0-0.8
Positive Control-CP (80 mg/kg body weight)	23.2*	N/A

* Statistically significant response using a one sample t-test

** MPCE in the concurrent vehicle control was 0 which is unusually low. So the data from female mice was compared to SITEK historical vehicle control data (Appendix II).

There was no evidence of a significant increase in the number of MPCE in the test article-treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups or SITEK's historical vehicle control data.

CP, the positive control, showed a statistically significant increase in MPCE (Tables 10 and 11). The mean numbers of MPCE in 2000 PCE were 28.8 and 23.2 for the male and female mice, respectively, thus fulfilling the criteria for a valid assay.

All remaining criteria for a valid assay were also met. In the vehicle control, the average number of MPCE per 2000 PCE did not exceed 10. Five animals from each sex were alive at the time of euthanasia for each dose level.

CONCLUSIONS

The test article, N,N,N',N'-tetramethyl ethanediamine (TMEDA, purity 99.86%), was tested for its potential to induce MPCE in the bone marrow cells of male and female CD-1 mice.

The results of this assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, the test article was negative in the Micronucleus Assay. It was concluded that the test article did not cause chromosome damage *in vivo* and, therefore is not considered to be a clastogenic agent.

REFERENCES

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2. OECD Guideline for the Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

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APPENDIX I

DATA TABLES

TABLE 1

RESULTS OF RANGE FINDING TEST

MOUSE BODY WEIGHTS INITIALLY AND APPROXIMATELY 72 HOURS FOLLOWING ORAL ADMINISTRATION OF TMEDA

STUDY NO.: 0977-1521 **VEHICLE: Water (10 mL/kg)**

MALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	5.0	10	50	100	200	300
	34	35	37	33	33	35	30
	35	35	34	34	33	35	36
	33	33	34	35	33	33	30
MEAN BODY WEIGHT	34	34	35	34	33	34	32

MALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	5.0	10	50	100	200	300
	34	36	36	34	33	35	32
	36	35	35	34	33	36	35
	34	33	35	36	33	33	**
MEAN BODY WEIGHT	35	35	35	35	33	35	34
% CHANGE MEAN BODY WEIGHT	2.9%	2.9%	0.0%	2.9%	0.0%	2.9%	6.1%

FEMALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	5.0	10	50	100	200	300
	27	29	27	28	27	27	24
	28	28	27	28	27	28	29
	26	27	26	26	27	29	26
MEAN BODY WEIGHT	27	28	27	27	27	28	28

FEMALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	5.0	10	50	100	200	300
	27	28	29	28	27	27	24
	29	29	27	30	26	27	30
	26	26	26	26	27	30	28
MEAN BODY WEIGHT	27	28	27	28	27	28	27
% CHANGE MEAN BODY WEIGHT	0.0%	0.0%	0.0%	3.7%	0.0%	0.0%	-3.6%

NOTE: Body weights are in grams. Only surviving animals are used for calculating %Change Mean Body Weight.

* Body Weights were taken prior to dosing.

** Animal found dead prior to 72 hours.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 2

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: MALE**

Test Article: TMEDA **DAY: 1**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity						3/3	3/3
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3		
Death							

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 3

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: FEMALE**

Test Article: TMEDA **DAY: 1**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity						3/3	3/3
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3		
Death							

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 4

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: MALE**

Test Article: TMEDA **DAY: 2**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							2/3
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	
Death							1/3

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level..

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 5

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: FEMALE**

Test Article: TMEDA **DAY: 2**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							3/3
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	
Death							

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 6

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: MALE**

Test Article: TMEDA **DAY: 3**

SIGNS	DOSE LEVELS (mg/kg)***						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	2/2**
Death							

* Vehicle used for this study was water (10mL/kg).
** One male died prior to Day 3.
*** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 7

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: FEMALE**

Test Article: TMEDA **DAY: 3**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 8

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: MALE**

Test Article: TMEDA **DAY: 4**

SIGNS	DOSE LEVELS (mg/kg)***						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	2/2**
Death							

* Vehicle used for this study was water (10mL/kg).
** One male dies prior to Day 4.
*** 3 mice per sex dosed at each dose level.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 9

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 0977-1521 **SEX: FEMALE**

Test Article: TMEDA **DAY: 4**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	5.0	10	50	100	200	300
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by QAU UL

Reviewed by Study Director JS

TABLE 10

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE
ORALLY ADMINISTERED TMEDA
24 HOURS AFTER TREATMENT

STUDY NO.: 0977-1521 **Vehicle: Water (10 mL/kg)**

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C2384	82	118	41.0	0	
VEHICLE	C2406	75	125	37.5	1	
VEHICLE	C2377	61	139	30.5	1	
VEHICLE	C2410	83	117	41.5	1	
VEHICLE	C2416	75	125	37.5	0	
	MEAN	75	125	37.6	0.6	
62.5	C2385	110	90	55.0	1	
62.5	C2413	79	121	39.5	0	
62.5	C2411	90	110	45.0	0	
62.5	C2421	100	100	50.0	0	
62.5	C2372	67	133	33.5	1	
	MEAN	89	111	44.6	0.4	*
125	C2419	72	128	36.0	0	
125	C2420	65	135	32.5	1	
125	C2418	83	117	41.5	0	
125	C2373	90	110	45.0	1	
125	C2415	51	149	25.5	2	
	MEAN	72	128	36.1	0.8	0.333
250	C2397	110	90	55.0	0	
250	C2392	75	125	37.5	0	
250	C2381	93	107	46.5	0	
250	C2402	78	122	39.0	1	
250	C2396	95	105	47.5	1	
	MEAN	90	110	45.1	0.4	*
CP**	C2423	30	170	15.0	30	
CP**	C2414	48	152	24.0	22	
CP**	C2407	45	155	22.5	30	
CP**	C2388	40	160	20.0	35	
CP**	C2389	40	160	20.0	27	
	MEAN	41	159	20.3	28.8	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 11

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE
ORALLY ADMINISTERED TMEDA
24 HOURS AFTER TREATMENT

STUDY NO.: 0977-1521 Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C2466	90	110	45.0	0	
VEHICLE	C2470	90	110	45.0	0	
VEHICLE	C2446	60	140	30.0	0	
VEHICLE	C2427	91	109	45.5	0	
VEHICLE	C2447	75	125	37.5	0	
	MEAN	81	119	40.6	0.0	
62.5	C2465	86	114	43.0	0	
62.5	C2460	72	128	36.0	1	
62.5	C2429	101	99	50.5	1	
62.5	C2468	115	85	57.5	1	
62.5	C2444	80	120	40.0	0	
	MEAN	91	109	45.4	0.6	0.385
125	C2459	58	142	29.0	0	
125	C2436	95	105	47.5	0	
125	C2438	91	109	45.5	0	
125	C2453	69	131	34.5	0	
125	C2474	86	114	43.0	0	
	MEAN	80	120	39.9	0.0	*
250	C2433	79	121	39.5	0	
250	C2425	72	128	36.0	1	
250	C2435	68	132	34.0	0	
250	C2448	88	112	44.0	1	
250	C2441	73	127	36.5	1	
	MEAN	76	124	38.0	0.6	0.385
CP**	C2461	70	130	35.0	16	
CP**	C2431	92	108	46.0	18	
CP**	C2450	95	105	47.5	26	
CP**	C2449	90	110	45.0	33	
CP**	C2432	86	114	43.0	23	
	MEAN	87	113	43.3	23.2	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by QAU _____ Reviewed by Study Director JS

TABLE 12

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE
ORALLY ADMINISTERED TMEDA
48 HOURS AFTER TREATMENT

STUDY NO.: 0977-1521 **Vehicle: Water (10 mL/kg)**

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE**
VEHICLE	C2387	108	92	54.0	1	
VEHICLE	C2417	128	72	64.0	1	
VEHICLE	C2394	120	80	60.0	0	
VEHICLE	C2376	131	69	65.5	1	
VEHICLE	C2374	104	96	52.0	1	
	MEAN	118	82	59.1	0.8	
62.5	C2375	95	105	47.5	1	
62.5	C2398	109	91	54.5	1	
62.5	C2409	80	120	40.0	2	
62.5	C2390	81	119	40.5	3	
62.5	C2382	101	99	50.5	4	
	MEAN	93	107	46.6	2.2	0.026
125	C2386	118	82	59.0	0	
125	C2378	121	79	60.5	0	
125	C2383	114	86	57.0	0	
125	C2380	115	85	57.5	0	
125	C2404	107	93	53.5	0	
	MEAN	115	85	57.5	0.0	*
250	C2401	95	105	47.5	2	
250	C2400	106	94	53.0	0	
250	C2405	102	98	51.0	0	
250	C2412	128	72	64.0	0	
250	C2422	108	92	54.0	0	
	MEAN	108	92	53.9	0.4	*

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.
** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by QAU VL

Reviewed by Study Director JS

TABLE 13

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE
ORALLY ADMINISTERED TMEDA
48 HOURS AFTER TREATMENT

STUDY NO.: 0977-1521 Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE**
VEHICLE	C2434	111	89	55.5	0	
VEHICLE	C2430	110	90	55.0	0	
VEHICLE	C2473	120	80	60.0	0	
VEHICLE	C2443	113	87	56.5	0	
VEHICLE	C2464	117	93	55.7	0	
	MEAN	114	88	56.5	0.0	
62.5	C2437	110	90	55.0	0	
62.5	C2451	110	90	55.0	0	
62.5	C2467	124	76	62.0	0	
62.5	C2445	121	79	60.5	0	
62.5	C2462	119	81	59.5	0	
	MEAN	117	83	58.4	0.0	*
125	C2439	105	95	52.5	0	
125	C2458	115	85	57.5	0	
125	C2426	110	90	55.0	0	
125	C2452	120	80	60.0	1	
125	C2440	118	82	59.0	0	
	MEAN	114	86	56.8	0.2	0.173
250	C2428	105	95	52.5	1	
250	C2471	97	103	48.5	1	
250	C2463	130	70	65.0	1	
250	C2454	118	82	59.0	0	
250	C2424	97	103	48.5	1	
	MEAN	109	91	54.7	0.8	0.173

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** The results are considered statistically significant if the p-value is less than or equal to 0.025.

TABLE 14

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Male Mice
Orally Administered TMEDA

Study No.: 0977-1521 **Vehicle: Water (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	75	125	37.6	-	0.6
24	62.5	89	111	44.6	18.6 %	0.4
24	125	72	128	36.1	-4.0 %	0.8
24	250	90	110	45.1	19.9 %	0.4
24	CP*	41	159	20.3	-46.0 %	28.8 **
48	Vehicle	118	82	59.1	-	0.8
48	62.5	93	107	46.6	-21.2 %	2.2
48	125	115	85	57.5	-2.7 %	0.0
48	250	108	92	53.9	-8.8 %	0.4

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

Reviewed by QAU UL

Reviewed by Study Director JS

TABLE 15

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Female Mice
Orally Administered TMEDA

Study No.: 0977-1521 **Vehicle: Water (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	81	119	40.6	-	0.0
24	62.5	91	109	45.4	11.8 %	0.6
24	125	80	120	39.9	-1.7 %	0.0
24	250	76	124	38.0	-6.4 %	0.6
24	CP*	87	113	43.3	6.7 %	23.2 **
48	Vehicle	114	88	56.5	-	0.0
48	62.5	117	83	58.4	3.4 %	0.0
48	125	114	86	56.8	0.5 %	0.2
48	250	109	91	54.7	-3.2 %	0.8

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

APPENDIX II

SITEK's HISTORICAL DATA FOR VEHICLE AND POSITIVE CONTROLS

MOUSE MICRONUCLEUS
HISTORICAL VEHICLE CONTROL DATA
MPCE per 2000 PCEs
October 2004 to April 2008

	MPCE/ 2000 PCE Males	MPCE/ 2000 PCE Females
Mean	0.5	0.5
Std. Dev.	0.41	0.37
Range	0 - 1.0	0 - 1.0
n=*	28	28

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

MOUSE MICRONUCLEUS
HISTORICAL POSITIVE CONTROL DATA
(CP 80 mg/kg)
MPCE per 2000 PCEs
October 2004 to April 2008

	MPCE/ 2000 PCE Males	MPCE/ 2000 PCE Females
Mean	40.2	43.2
Std. Dev.	13.38	14.55
n=*	16	16
Range	18.2 - 76.6	19.6 - 71.8

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

APPENDIX III

STUDY PROTOCOL, PROTOCOL AMENDMENT AND DEVIATIONS

SITEK RESEARCH LABORATORIES

IN VIVO TEST FOR CHEMICAL INDUCTION OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES IN MOUSE BONE MARROW CELLS

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign Section 10 to approve the protocol.**

PART ONE

1.0 SPONSOR

Name: USA RDECOM, AMSRD-MSF

1.2 Address: Environmental Acquisition & Logistics Sustaining Program

Aberdeen Proving Ground, MD 21010

1.3 Sponsor's Study Coordinators: Gunda Reddy, Ph.D., DABT

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Shambhu Kumar Roy, Ph.D.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 0977-1521

3.2 Sponsor's Study No.: Not available

4.0 TEST ARTICLE

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

4.1 Identification

Name: TMEDA (N,N, N'N'-tetramethyl ethanediamine) (CAS Registry No. 110-18-9)

Batch/Lot No.: Not provided

4.2 Description

Color: Clear

Physical Form: Liquid

4.3 Analysis

Purity Information: Not provided

Does the Sponsor require the use of a correction factor to account for impurity?

____ Yes __X__ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

__ Room Temperature __X__ Refrigerated (1-5°C)

__ Frozen (-10 to -20°C)

__ Other (please specify): _____

Expiration Date: Provide by Sponsor SKR entered le JS 4-22-08

4.5 Preferred Vehicle (check one):

__X__ H_2O ____ Saline ____ Corn Oil ____ DMSO ____ Ethanol(50% v:v in H_2O)

____ Other (please specify): _____

____ To be decided by the Testing Facility

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4.6 Special Handling Instructions:

Take all safety precautions followed when working with hazardous Substances. See the MSDS.

5.0 TEST ANIMALS AND TEST ARTICLE ADMINISTRATION

5.1 Test Animals (please check):

5.1.1 For the Range Finding Test.

____ Males ____ Females __X__ Both

5.1.2 For the Micronucleus Assay.

____ Males ____ Females __X__ Both

5.2 Route of Test Article Administration (check one):

_____ IP Injection __X__ Oral Gavage

The test article and vehicle will be administered by IP injection or oral gavage as indicated above, either directly or through a vehicle compatible with the test system. These routes of administration are valid methods for introduction of the test article. If necessary, other appropriate methods can be used at the request of the Sponsor.

_____ Other ** (please specify): _____

5.3 Frequency of Test Article and Vehicle Control Administration (check one):

__X__ Single administration and multiple harvests (approximately ____ 24, 48 and 72 hours or __X__ 24 and 48 hours after dose administration) (check one).

_____ Multiple administrations on ___ 2 or ___ 3 (check one) consecutive days and single harvest (approximately 24 hours after the last dose administration).

5.4 Volume of Administration

4.0 mL/kg will be administered when DMSO is used as the vehicle. 10-20 mL/kg will be administered when water, saline, corn oil, or other nontoxic materials are used as the vehicles.

** Additional charges will apply.

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6.0 REGULATORY AGENCY SUBMISSION

6.1 Test Design Specifications

This study protocol is designed to meet or exceed the U.S. EPA, ICH and OECD Guidelines specified in the following documents (1, 2, 3):

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

6.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45[ENV/MC/CHEM (98)17], Paris 1998.

Will this study be submitted to a regulatory agency?

_X_ Yes **__ No**

If so, which agency(ies)? Worldwide

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7.0 DOSING SOLUTIONS

The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; EPA FIFRA = 40 CFR, 160.113, and OECD GLPs, Section 6.2.

Does the Sponsor want dosing solution analysis?

____ Yes** **__X__ No**

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and/or stability of the dosing solutions. Stability will only be determined if the dosing solutions are not prepared immediately prior to each use. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and/or stability of the dosing solutions.

Dosing solution analysis will be performed by:

____ SITEK Research Laboratories **____ Sponsor*****

What dosing solutions will be analyzed?

From the Range Finding Test?

____ Yes **____ No**

From the Assay?

____ Yes **____ No**

Which concentration(s)? _____

** Additional charges will apply. See Special Services price schedule.

***Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:**

- **All subcontract and/or Sponsor Quality Assurance audit findings and comments.**
- **Any deviations and/or amendments, if applicable.**
- **An original or copy of the analysis report.**
- **Location (address) of where the raw data from the analysis will be archived by the Sponsor or Subcontractor.**

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.

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What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ **Room Temperature** _____ **Frozen (-10 to -20 °C)**

_____ **Refrigerated (1-5 °C)**

At what temperature should the dosing solutions be shipped?

_____ **Room Temperature** _____ **On Wet Ice**

_____ **On Dry Ice**

8.0 BLOOD (PLASMA) ANALYSIS FOR TEST ARTICLE CONCENTRATIONS

Does the Sponsor want blood (plasma) analysis?

_____ **Yes**** __X__ **No**

Blood (plasma) analysis will be performed by:

_____ **SITEK Research Laboratories** _____ **Sponsor**

What blood (plasma) analysis will be performed?

From the Range Finding Test?

_____ **Yes** _____ **No**

From the Assay?

_____ **Yes** _____ **No**

Test Animals: Males only.

Time points (bleeding time after the dose administration), dose levels and number of animals for the blood sample collection (unless otherwise specified by the Sponsor).

From the Range Finding Test (if sufficient pharmacokinetic information is not available), blood samples will be taken after dose administration at 3 time points (to be determined by the Sponsor), at 3 dose levels starting from the highest dose, then every other dose level. One male per dose per time point and a total of 9 males will be used.

** Additional charges will apply. See Special Services price schedule.

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From the Micronucleus Assay blood samples will be taken from all three dose levels. Only 1 time point will be used based on the plasma analysis results from the Range Finding Test or on the pharmacokinetic information of the test article (to be determined by the Sponsor). Three males per dose per time point and a total of 9 males will be used.

If requested by the Sponsor, SITEK Research Laboratories will determine the test article concentration in the plasma. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

9.0 STUDY DATES

* 9.1 Proposed Experimental Start Date: March 12, 2008

Defined as the date the animals are dosed for the Range Finding Test or Micronucleus Assay.

* 9.2 Proposed Experimental Completion Date: April 26, 2008

Defined as the last date on which data are collected directly from the study.

* 9.3 Proposed Draft Report Date: May 31, 2008

9.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

10.0 PROTOCOL APPROVAL

* _____ 1·23·08
Study Director Date

_____ 1-15-08
Sponsor's Study Coordinator Date

* _____ 1/23/08
Quality Assurance Manager Date

* _____ 1/23/08
Safety Officer Date

* To be completed by the Testing Facility.

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STUDY DESIGN

PART TWO

11.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes in mouse bone marrow cells (4,5).

12.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (6, 7, 8).

13.0 ABBREVIATIONS

CP	-	Cyclophosphamide
DMSO	-	Dimethyl Sulfoxide
FBS	-	Fetal Bovine Serum
IACUC	-	Institutional Animal Care and Use Committee
IP	-	Intraperitoneal
MPCE	-	Micronucleated Polychromatic Erythrocytes
MTD	-	Maximum Tolerated Dose
NCE	-	Normochromatic Erythrocytes
PCE	-	Polychromatic Erythrocytes

14.0 ANIMALS

14.1 Protocol Approval

This protocol has been approved for the necessity of using laboratory animals by SITEK's Institutional Animal Care and Use Committee (IACUC) on February 24, 2006 (No. 06-02). In this regard, the number of animals used, the objectives of the study, the housing procedures, the rationale for animal use, the procedures involving animals, the potential for distress or pain for the animals, and the methods of euthanasia have been reviewed and approved.

14.2 Source

Forty-two-day-old (approximately), male and female CD-1 mice will be obtained from Harlan Sprague Dawley Inc. or other acceptable vendor which routinely monitor the animals for bacterial, viral and other murine infections. The animal body weight range will be approximately 16-38 grams on delivery.

14.3 Housing and Quarantine

When the animals arrive, they will be housed according to sex in clean cages of sufficient size to allow free movement. They will be placed, one sex at a time, up to ten animals per cage, into polycarbonate cages. Hardwood chip bedding free of injurious substances will be used. The animals will receive Purina Certified Rodent Diet and fresh tap water ad libitum. The levels of contaminants present in the food and water are very low and well within acceptable levels, and are not expected to affect the outcome of the study. Bedding will be changed at least twice a week.

The animals will be quarantined for at least 7 days prior to dose administration. The animals will be observed each day, and all observations and the temperature and humidity of the animal room will be recorded in the study notebook. The animal room will be maintained at 18-26 °C and 30-70% humidity. Occasionally, minor deviations may occur due to change of outside weather conditions. A 12-hour diurnal light cycle will be employed. Only animals that are certified as healthy will be used in the study.

15.0 TEST SYSTEM IDENTIFICATION

All animals to be treated will receive an ear tag with a number unique to the particular study. All cages will be assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director, and the animal study proposal number approved by SITEK's IACUC. The microslides will be labeled with the study number, the last one, two, three or four digits of animal number as code number and date of slide preparation.

16.0 CONTROL SUBSTANCES

16.1 Positive Controls

Cyclophosphamide (CP), at 80 mg/kg (8.0 mg/mL X 10 mL/kg) as a single dose by oral gavage, will be dissolved in water and used as the positive control. The source and storage conditions for CP are given below:

Source: Sigma Chemical Company or other vendor Storage Conditions: 1-5°C CAS No.: 6055-19-2

The specific source, lot number and expiration date of the CP will be documented in the report.

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

16.2 Vehicle Controls

The vehicle used for dissolving or suspending the test article will be used as the vehicle control. Deionized, distilled water, saline, corn oil (CAS #80001-30-7), dimethyl sulfoxide (CAS #67-68-5), methyl cellulose (CAS 9004-67-5), hydroxypropylmethyl cellulose (CAS #9004-65-3), tween 80 (CAS #9005-65-6) and ethanol (CAS #64-17-5) are some of the vehicles, which are compatible with this test system. If these vehicles are not suitable, others may be used with the approval of the Sponsor. The source, lot number and storage conditions of the vehicle control will be documented in the report.

17.0 DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study will be entered in a study notebook. The study notebook will also include the protocol, protocol amendments and deviations, copy of the study report, and all relevant communications with the Sponsor.

18.0 EXPERIMENTAL PROCEDURE

18.1 Determination of Solubility/Miscibility

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test will be performed.

The test article will be tested for its solubility/miscibility in deionized, distilled water, saline, DMSO, corn oil, ethanol and/or other appropriate vehicles. Solid and viscous liquid test articles will be tested for solubility in weight per volume, and nonviscous liquids will be tested for miscibility in volume per volume or weight per volume. The solubility/ miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test will consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of vehicle has been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of vehicle required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test will be conducted. 1.5 mL of vehicle in 0.1 mL increments will be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in 0.5 mL increments until 5.0 mL has been added. The resulting solution will be thoroughly mixed

and observed for miscibility. The test article will be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations will be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article will be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data are available, the solubility/miscibility test will not be performed.

18.2 Randomization

The animals will be randomized one sex at a time and placed into experimental groups as follows: In the Range Finding Test, the animals will be randomly assigned to experimental groups, without regard to body weight, prior to dose administration (three animals per group). In the Micronucleus Assay, the animals will be weighed and placed in weight groups of 1-gram differences. Using a computer generated random number matrix, the animals will be assigned sequentially, from low to high weight groups, to cages corresponding to treatment groups (five animals per group).

18.3 Preparation of Test Article

The stock solutions for the Range Finding Test and the Micronucleus Assay will be prepared as specified in the dilution scheme which will be kept in the study notebook. The highest stock solution will be prepared by mixing together the required weight/volume of the test article and the appropriate amount of the vehicle until complete solubilization or a homogeneous suspension has been achieved. The remaining doses specified in the dilution scheme will be prepared as in the case of the highest stock solution, by performing a subsequent dilution or by varying the volume administered from the highest stock concentration to the animals. When preparing the top dosing stock and any subsequent dosing stock with a viscous or non-viscous liquid, the test article should never be diluted more than 10-fold. The dosing solutions will be prepared by SITEK study personnel just prior to treatment and kept at room temperature. In all treatments, the amount of vehicle administered to the animals will be limited to a level which has no significant toxic effect. If necessary, the test article may be administered at full strength.

18.4 Range Finding Test

The dose levels for the Micronucleus Assay will be selected according to the toxicity of the test article and in consultation with the Sponsor. If sufficient information is not available on the toxicity of the test article, a Range Finding Test will be performed. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. Unless otherwise specified by the Sponsor, the doses for the Range Finding Test are 2000, 1000, 500, 100, 50 and 10 mg/kg for solid and viscous test articles and 2.0, 1.0, 0.5, 0.1, 0.05 and 0.01 mL/kg for liquid test articles.

Treatment groups of three animals per sex will be dosed with the test article dosing solutions using the route, frequency and volume of administration selected in Section 5.0. One treatment group will be treated with only the vehicle. Food and water will be provided ad libitum. The animals will be carefully observed for 3 days after the single or the first administration of the test article. Daily records of all clinical observations and number of deaths (if any) will be kept for the treatment groups. Body weights will be checked on the

day of dosing (Day 1) and by the end of the 3-day observation period (Day 4). All animals will be euthanized at the end of the observation period. The body weights will be entered into a computer, using MS Excel 97 Spreadsheet program. The clinical signs and mortality will be entered into a computer, using MS Word 97.

In addition to the animals treated with the test article for the standard Range Finding Test, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Range Finding Test.

The animals will be euthanized by CO_2 asphyxiation. Blood will be taken by cardiac puncture.

Approximately 0.4-1.0 mL samples of blood will be drawn into a syringe containing heparin (10,000 units). The needles will be removed from the syringes, and the blood will be transferred to an appropriately labeled 1.5 mL microfuge tubes. The tubes will be capped and placed on wet ice. Labeling will include Study number, test article identification, dose level, animal number and sampling time.

Within 0.5 hour of collection, the samples will be centrifuged at a speed of 14,000 rpm for 2 minutes. Being careful not to transfer any of the red blood cell fractions, the plasma fractions will be transferred by pipet into 1.5 mL storage vials equipped with O-ring screw caps. The samples will be stored immediately at -10 to -20°C. The frozen plasma samples will be shipped overnight on dry ice.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma in order to pick the time point for the Micronucleus Assay.

The toxicity of the test article will be evaluated on the basis of a combination of factors, namely, number of deaths, loss of body weight, and other clinical symptoms observed during the 3-day period. If possible, the highest dose selected for the Micronucleus Assay will be the Maximum Tolerated Dose (MTD) at which no deaths are recorded but animals show evidence of toxicity and/or more than 10% loss of body weight. If no toxicity is observed, the maximum dose treated will be 2000 mg/kg or 2.0 mL/kg. In addition, two lower doses, preferably one-half and one-fourth of the high dose, will be included in the assay.

18.5 Micronucleus Assay

The animals will be randomized and placed into treatment groups of five males and/or five females. One or three treatment groups will be assigned to each dose level and vehicle control. The dose levels will be as determined by the Range Finding Test after consultation with the Sponsor. One treatment group will be assigned to the positive control.

Food and water will be provided ad libitum. The test article will be administered as indicated in Section 5.0. Appropriate vehicle and positive controls will be maintained.

The animals will be euthanized by CO_2 asphyxiation. One treatment group from each test article dose level and vehicle control will be euthanized approximately 24 and 48 hours or 24, 48 and 72 hours after dose administration. Only one euthanasia, 24 hours after the

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dose administration, will be made of the animals dosed with the positive control. If multiple administrations and single harvest are selected, the animals will be euthanized approximately 24 hours after the last dose administration.

The number of animals to be used in the standard Micronucleus Assay will be as follows (if both sexes are used):

If single administration and multiple harvests are used:

For three harvests:

	No. of Animals Per Harvest Time						
Dose Level	24 Hours		48 Hours		72 Hours		
	Males	Females	Males	Females	Males	Females	Total
High	5	5	5	5	5	5	30
Mid	5	5	5	5	5	5	30
Low	5	5	5	5	5	5	30
Vehicle Control	5	5	5	5	5	5	30
Positive Control	5	5	N/A	N/A	N/A	N/A	10
							130

For two harvests:

	No. of Animals Per Harvest Time				
Dose Level	24 Hours		48 Hours		
	Males	Females	Males	Females	Total
High	5	5	5	5	20
Mid	5	5	5	5	20
Low	5	5	5	5	20
Vehicle Control	5	5	5	5	20
Positive Control	5	5	N/A	N/A	10
					90

If multiple administrations and single harvest are used:

	No. of Animals Per Group		
Dose Level	Males	Females	Total
High	5	5	10
Mid	5	5	10
Low	5	5	10
Vehicle Control	5	5	10
Positive Control	5	5	10
			50

After the animal has been euthanized, the groin area will be cleansed with 70% ethanol, and both femurs will be exposed by cutting into the skin and muscle of the thighs. The femurs will be separated just above the kneecaps, and the femur heads will be removed

with scissors. The bone marrow from both femurs will be flushed into a medium disposable culture tube (13x100 mm), containing 1.0 mL of FBS, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes will be centrifuged at 800 rpm for 5 minutes, and the supernatant will be removed, leaving approximately 0.1 mL of serum above the cell pellet. The cell pellet will be resuspended in the remaining serum until a homogeneous suspension is observed.

A small drop of the cell suspension will be placed on the unfrosted end of a clean microslide and spread along the length of the slide. The slides will be allowed to air dry, fixed in methanol for 15 minutes, and allowed to air dry again. The slides will be stained in Wright-Giemsa stain for 2-4 minutes, rinsed in distilled water, allowed to air dry completely, and mounted in Permount using #1 cover glasses. The backs of the slides will be cleaned with methanol.

The slides will be scored "blind" in order to avoid bias on the part of the scorer(s). The number of PCE among total erythrocytes (PCE + NCE) will be determined for each animal by counting a total of at least 200 erythrocytes. The number of MPCE then will be scored for 2000 PCE per animal (2).

In addition to the animals treated with the test article for the standard Micronucleus Assay, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Micronucleus Assay.

The animals will be euthanized by CO_2 asphyxiation. Blood will be taken by cardiac puncture.

The procedures for blood collection, storage and shipping will be the same as in the Range Finding Test.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma. There is sufficient correlation for measurements of test article in plasma to be adequate for validating bone marrow exposure (3).

18.6 Statistical Analysis

The data will be analyzed separately for male and female animals. The data from the score sheets will be consolidated into summary sheets and entered into a computer using a MS Excel 97 validated spreadsheet program. The treatment group means will be calculated for the percentage of PCE among total erythrocytes, as well as the frequency of MPCE. A significant reduction (more than 20% versus that of the control vehicle) will be used as an indication of toxicity. Unless otherwise indicated, the frequency of MPCE in each treatment group will be compared to that in the respective vehicle control using a one-tailed Student's t-test (9). Epistat statistical package will be used to calculate p values for the t-test. The results will be considered significant if the p value is ≤ 0.025. The Cochran-Armitage Test (trend test) (10) will be used for evidence of a dose-related response, if the Student's t-test shows a positive result. The trend test will be considered significant if the p value is ≤ 0.05.

Historical vehicle control values also will be taken into consideration when necessary as described in Section 18.8.1. Statistical analysis will not be performed if the test dose value is equal to or less than the concurrent or historical vehicle control.

18.7 Criteria for a Valid Assay

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed ten.

2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.

3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

18.8 Evaluation of Test Results

18.8.1 Positive Response

The test article will be considered to have caused a positive response in this assay if:

1. The test article shows a positive dose-response trend and a statistically significant increase ($p \leq 0.025$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article causes a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may be compared to historical vehicle control data.

2. In the event there is no positive dose-response trend, at least two consecutive test doses show a statistically significant increase in the number of MPCE.

18.8.2 Negative Response

The test article will be considered to have caused a negative response if none of the test doses show a statistically significant increase in the number of MPCE when compared to the vehicle control.

18.8.3 Equivocal Response

If the test article induces a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend, the results will be considered equivocal. In such a case, a repeat assay will be performed with the approval of the Sponsor.

18.8.4 Other Considerations

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

19.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor, and these changes and the reasons for them will be documented. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and these changes, along with the reasons for them or explanations, will be documented. If the amendments or deviations involve animal use, they will be reviewed by the IACUC. Protocol amendments and deviations will be appended to the protocol.

20.0 REPORT OF RESULTS

20.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study, and the dates on which the study was initiated and completed.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Statistical methods employed for analyzing the data.

4. The test and control articles identified by name, chemical abstracts number or code number, strength, purity and composition, or other appropriate characteristics.

5. A description of the methods used.

6. The name, sex and source of the animals used.

7. A description of the treatment procedures, vehicle used for treatment and duration of treatment.

8. A description of all circumstances that may have affected the quality or integrity of the data.

9. The name of the Study Director and the names of other technical personnel or other professionals, who participated in performing the study.

10. A description of the transformations, calculations or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

11. The signed and dated final report of the Study Director and Quality Assurance Manager.

12. The location where the raw data and final report are to be stored.

13. A statement from the Quality Assurance Unit.

20.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments and will include the reasons for the changes. Report amendments will be added to the final report as an addendum.

21.0 ARCHIVES

The raw data, documentation, protocol and Final Report, along with an electronic file containing the data tables and copy of the Final Report of the study will be maintained in SITEK Research Laboratories' Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, for ten years. All raw data, documentation, and the final report of all Subcontractors/Sponsor work will be maintained by the Subcontractor/Sponsor.

22.0 REFERENCES

1. United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

2. OECD Guideline for the Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

4. Heddle, J. A. A rapid in vivo test for chromosomal damage. Mutat. Res., 18, 187-190, 1973.

5. Schmid, W. The micronucleus test. Mutat. Res., 31, 9-15, 1975.

6. Mavournin, K. H., et al. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res., 239, 29-80, 1990.

7. Hayashi, M., et al. In vivo rodent erythrocyte micronucleus assay. Mutat. Res., 312, 293-304, 1994.

8. Tice, R. R. and M. D. Shelby. Report of in vivo subgroup. Mutat. Res., 312, 287-292, 1994.

9. Lovell, D. P., et al. Statistical analysis of in vivo cytogenetic assays in: D. J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge. pp. 184-232, 1989.

10. Margolin, B. H., et al. Statistical analysis for the in vitro cytogenetic assay using Chinese hamster ovary cells. Enviro. Mutagen., 8, 183-204, 1986.

PROTOCOL AMENDMENT

Amendment No.:	1
Sponsor:	USA RDECOM, AMSRD-MSF, Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0977-1521
Sponsor's Study No.:	N/A
Test Article I.D.:	N,N,N',N'-tetramethyl ethanediamine (TMEDA)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells
Amendment No. 1:	Protocol Page 1, Section 2.3. The study director has been changed from Dr. Shambhu K. Roy to Dr. Jian Song.
Reason for Amendment No. 1:	Dr. Shambhu K. Roy is no longer employed by SITEK Research Laboratories.

APPROVED:

Paul E. Kirby, Ph.D.
President

6-18-08
Date

PROTOCOL AMENDMENT

Amendment No.:	2
Sponsor:	USA RDECOM, AMSRD-MSF, Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0977-1521
Sponsor's Study No.:	N/A
Test Article I.D.:	N,N,N',N'-tetramethyl ethanediamine (TMEDA)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells
Amendment No. 2:	Protocol Page 11, Section 18.4, The doses for the Range Finding Test have been changed from 2000, 1000, 500, 100 50 and 10 mg/kg to 300, 200, 100, 50, 10 and 5.0 mg//kg. The doses for the Micronucleus Assay were 250, 125 and 62.5 mg/kg.
Reason for Amendment No. 2:	Toxicological information from MSDS indicated the LD50 of oral rat was 268 mg/kg. Protocol Page 11, Section 18.4. The actual doses for the assay, once determined, will be added to the protocol in the form of an amendment.

APPROVED:

Jian Song, Ph.D.
Study Director

6-18-08
Date

PROTOCOL DEVIATION

Deviation No.:	1
Sponsor:	USA RDECOM, AMSRD-MSF, Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0977-1521
Sponsor's Study No.:	N/A
Test Article I.D.:	N,N,N',N'-tetramethyl ethanediamine (TMEDA)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells
Deviation No. 1:	Protocol Page 9, Section 14.3 Housing and Quarantine: As per protocol, the animal room should have been maintained at 18-26°C and 30-70% humidity. However, the temperature range was 17-23°C and the humidity range was 17-54%.
Reason for Deviation No. 1:	This deviation occurred due to changes in outside weather conditions. However, this deviation does not affect the outcome of the study.

APPROVED:

Jian Song, Ph.D.
Study Director

6-18-08
Date

APPENDIX IV

CERTIFICATE OF ANALYSIS

SIGMA-ALDRICH

Certificate of Analysis

Product Name	*N,N,N′,N′*-Tetramethylethylenediamine, ≥99.5%, purified by redistillation
Product Number	411019
Product Brand	Aldrich
CAS Number	110-18-9
Molecular Formula	$(CH_3)_2NCH_2CH_2N(CH_3)_2$
Molecular Weight	116.20

TEST	SPECIFICATION	LOT 10588KD RESULTS
APPEARANCE	COLORLESS LIQUID	COLORLESS LIQUID
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
GAS LIQUID CHROMATOGRAPHY	99.50% (MINIMUM)	99.86%
COLOR TEST	20 APHA (MAXIMUM)	<10 APHA
QUALITY CONTROL ACCEPTANCE DATE		SEPTEMBER 2005

Barbara Rajzer, Supervisor
Quality Control
Milwaukee, Wisconsin USA